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ORIGINAL ARTICLE

Development of *Anncaliia algerae* (Microsporidia) in *Drosophila melanogaster*

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ABSTRACT

Representatives of the genus *Anncaliia* are known as natural parasites of dipteran and coleopteran insects, amphipod crustaceans, but also humans, primarily with immunodeficiency. *Anncaliia algerae*-caused fatal myositis is considered as an emergent infectious disease in humans. *A. (=Nosema, Brachiola) algerae*, the best studied species of the genus, demonstrates the broadest among microsporidia range of natural and experimental hosts, but it has never been propagated in *Drosophila*. We present ultrastructural analysis of development of *A. algerae* in visceral muscles and adipocytes of *Drosophila melanogaster* 2 wk after per oral experimental infection. We observed typical to *Anncaliia* spp. features of ultrastructure and cell pathology including spore morphology, characteristic extensions of the plasma membrane, and presence of "ridges" and appendages of tubular material at proliferative stages. *Anncaliia algerae* development in *D. melanogaster* was particularly similar to one of *A. algerae* and *A.(Brachiola) vesicularum* in humans with acute myositis. Given *D. melanogaster* is currently the most established genetic model, with a fully sequenced genome and easily available transgenic forms and genomic markers, a novel host–parasite system might provide new genetic tools to investigate host–pathogen interactions of *A. algerae*, as well to test antimicrosporidia drugs.

A microsporidium *Anncaliia (syn. Brachiola, Nosema) algerae* (Vavra and Undeen 1970) (Cali et al. 1998; Franzen et al. 2006a,b; Issi et al. 1993) was first described from a laboratory colony of mosquitoes *Anopheles stephensi* (Vavra and Undeen 1970). This microsporidium belongs to the *Anncaliia-Tubulinosema* clade that contains species with a broad host range among arthropods, some of which tolerate high temperatures, usually critical for microsporidia. Preadaptation to development at high temperatures might partly explain the fact that the species of this clade develop opportunistic infections in humans (Cali et al. 2005; Choudhary et al. 2011; Meissner et al. 2012; Sutrave et al. 2018; Watts et al. 2014) more often than representatives of other Microsporidia taxa. The genus *Anncallia* contains members naturally parasitic to invertebrates, namely insects of the orders Diptera (*A. gambiae* (Weizer, Zizka 2004), *A. algerae* (Andreadis 2007)), and *Coleoptera* (*A. varivestis* (Brooks et al. 1985), *A. meligethi*; (Issi et al. 1993)), and to amphipod crustaceans *(Anncaliia azovica* (Tokarev et al. 2018)). Two species, *A. connori* and *A. vesicularum,* were discovered only in humans with immunodeficiency (Cali et al. 1998), and their environmental source is unknown (Weiss 2014). Among *Anncaliia* spp., *A. algerae* is an example of a truly generalist microsporidium. In addition to being a common parasite of several genera of mosquitoes (Andreadis 2007), it was occasionally reported to cause eye, skin, and surface muscle infections transmitted presumably by a mosquito vector, in immunologically healthy individuals (Visvesvara et al.1999; Weiss 2014). In immunocompromised humans, particularly in HIV-positive patients, *A. algerae* cause severe infections in brain, eyes, muscles, as well as disseminated disease (Weiss 2014). But the most attention *A. algerae* has recently attracted is as an emerged cause of human myositis. Eight cases of *A. algerae*-associated myositis (of which five were fatal), were described in

patients who had taken immunosuppressive medications for rheumatoid arthritis, diabetes or organ transplantation (Anderson et al. 2019; Coyle et al. 2004; Sutrave et al. 2018; Watts et al. 2014). The abovementioned information allows us to consider *A. algerae* an important human pathogen. This microsporidium is also known to develop infections in SCID mice (Koudela et al. 2001) and can be grown in insect (Visvesvara et al. 1999), fish (Monaghan et al. 2011), and mammalian (Trammer et al. 1999) cell lines.

For the first time, we demonstrate here *A. algerae* infection in *Drosophila melanogaster*. We also present ultrastructural analysis of development of *A. algerae* in muscles and adipocytes of *D. melanogaster,* two weeks after per oral experimental infection. Association of *A. algerae* and *D. melanogaster* might exemplify one more system in which a microsporidium infects a model organism, in addition to *Danio rerio—Pseudoloma neurophilia* (Matthews et al. 2001; Ramsay et al. 2009) and *Caenorhabditis elegance—Nematocida parisii* (Troemel et al. 2008). The importance of such systems based on knowledge of host genomics and proteomics, and accessibility of host genomes to manipulations, cannot be overestimated (Troemel 2011). In particular, existence of numerous *Drosophila* strains that express fluorochrome-tagged proteins allows visualization of participation of these proteins in host–parasite interactions including their incorporation in parasite cells. For example, current research demonstrated fluorescence on the surface of spores after a passage through *Drosophila* strain that expresses green fluorescent protein (GFP)-tagged E-cadherin (see Fig. 1B, S1 and Comment 1 in Supporting Information).

MATERIALS AND METHODS

Source of flies and microsporidia

Fly stock #58471 from the Bloomington Drosophila Stock Center at Indiana University (https://bdsc.indiana.edu/) expressed GFP-tagged E-cadherin (w[*]; P{w[+mW.hs]=FRT(w[hs])}G13 shg[1]/CyO; P{w[+mC]=Ubi-p63E-shg.GFP}3). Flies were raised at 23–25 °C on standard cornmeal molasses agar medium supplemented with granular yeast or yeast paste. Spores of *Anncaliia algerae* were obtained from James J. Becnel (USDA ARS, Gainesville). Approximately 5×10^6 spores were suspended in 200 μl of distilled water and applied onto a thin film of yeast covering the agar cushion in the vial with eggs and freshly hatched larvae. *Drosophila* development from the egg to imago lasted 11–16 d at 25 °C (egg, 1–2 d, larvae, 6–8 d, pupae, 4–6 d). So at the time of observation (21–26 dpi), all hatched larvae had developed into imagoes. The latter were tested for microsporidiosis. Females and males were differentiated from each other by shape and color of genitalia (black and rounded in males vs. light and pointed in females), as well as by presence (males) or absence (females) of sex combs on the 4th segment of front legs. To test the progeny for vertically transmitted infection, the remaining flies were transferred to a fresh vial with diet and allowed to lay eggs.

Light microscopy

Infection was detected in imagoes 3–4 wk postinfection by observation of spores in tissue smears. The smears were obtained from flies dissected in 2% paraformaldehyde, stained with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI) or Calcofluor-white (1.0 μg/ml in the same fixative), or Giemsa stain, and examined in Zeiss Axioskop equipped for phase contrast and fluorescence microscopy (Thornwood, NY), along with a SPOT SE camera and software for image acquisition (Diagnostic Instruments, Sterling Heights, MI).

Confirmation of the identification of the parasite species by PCR

Because other microsporidia are known to infect *D. melanogaster* (Franzen et al. 2005, 2006a,b; Kramer 1964), and experimental infections may provoke benign infections with natural microsporidia, we confirmed the species identification for the spores used for infection and those that had passed through flies, by PCR using universal microsporidian primers V1-1492 (Vossbrinck et al. 2004). The 1,200 bp long PCR products were identical to each other and exactly matched the orthologue isolated from *Anopheles stephensi* and deposited in Gene Bank under Accession #AF069063.1

For electron (EM) and light microscopies (LM)

Small pieces of tissues were fixed in a mixture of 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate buffer supplemented with 5% sucrose for 2 h, washed several times in the same buffer, and postfixed in 1% osmium for 1 h, at room temperature. Samples were then thoroughly washed in water and dehydrated in ascending ethanol series and propylene oxide, and embedded in Epon-Araldite. Blocks were sectioned with Ultratome Leica EM UC7. Thick (0.5–1 μm) sections were stained with the modified Methylene blue stain and examined in Zeiss Axioplan microscope. Only those blocks that contained identifiable microsporidian spores or other stages were subjected for subsequent EM examination. Thin (70–80 nm) sections were stained with uranyl acetate and lead citrate and examined in JEOL JEM 1011 microscope with the attached HAMAMATSU ORCA-HR digital camera. All reagents for LM were from SIGMA-ALDRICH (St. Louis, MO) and for EM from EMS Chemicals (Fort Washington, PA).

RESULTS AND DISCUSSION

Infection experiments

All together four attempts to infect flies were undertaken. In the first two experiments, about 1/3 of tested insects

got infected, but the exact counts were not performed. In the two last experiments, infection rates were recorded more accurately (Table S1). Overall infection rate in these experiments was 21% and 29% based on observation of microsporidian spores in images 21–26 d postinfection (dpi). Spores were observed in imagoes of both sexes. At the same time, we never observed spores in larvae, which can be explained either by a long life cycle of the parasite or/and by the presence of a putative factor(s) that prevents spore maturation at preimaginal phases but triggers it in spores. Interestingly, that spores of some other *Anncaliia* spp. naturally infecting insects were also produced exclusively in imagoes (Brooks et al. 1985; Issi et al. 1993). Low infection of the second generation suggests that the parasite was not efficiently transmitted vertically.

Light microscopy

There were no visible signs of gross pathology. Infection could be detected only by observation of spores (Fig. 1A, B) and stages in the microscope. Staining the smears from infected flies with DAPI (Fig. 1A) and Giemsa (Fig. S1b, c) revealed diplokarya in all observed proliferative stages and spores. Live spores extruded polar filaments upon desiccation (Fig. S1a). The length of extruded filaments was 65–85 μm. Spores were ovoid with one end slightly more pointed. Live spores averaged (±SE): $3.54 \pm 0.035 \times 2.10 \pm 0.025$ μm, $n = 40$ and ranged: 3.15–4.04 × 1.74–2.49 μm, which is in a good accord with original description of *Nosema algerae* by Vavra and Undeen (1970). Numerous mature and immature spores were visible in smeared muscles (Fig. S1c).

Electron microscopy

The following tissues were examined: intestine, peripheral fat body, ovaries, and skeletal musculature. The infection was detected only in the blocks containing intestine and ovaries. However, neither gut epithelium, nor ovarioles per se were infected. *Anncaliia algerae* proliferative stages and spores were found mostly in the circular fibers of the visceral musculature that envelops the midgut (Fig. 1C, G–J, 2G and Fig. S2c, d), but also in the fat body surrounding ovaries (Fig. 1D–F and Fig. S2a, S3a–e), and in a few tracheoles in vicinity of the midgut (Fig. S2b). Noteworthy, that adult visceral musculature descends from larval visceral fibers that persist metamorphosis (Aghajanian et al. 2016), unlike most other larval tissues and organs including gut epithelium and skeletal musculature. Circular fibers thus were the site, the closest to the gut, suitable for parasite development. Interestingly, that even longitudinal fibers which form the next after circular fibers layer enveloping the gut were not infected, or at least were much less infected, below the threshold allowing visualization. The earliest stages observed in all infected tissues were diplokaryotic proliferative stages with thickened plasmalemma bearing characteristic for *Anncaliia* spp. tubular structures on the surface (Fig. 1C–E, 2A–C and Fig. S2b, S3a, b). These stages underwent multiple division by mitosis (Fig. 1E). Karyokinesis and cytokinesis were linked, because only diplokaryotic cells were observed. Some presumably earlier proliferative stages (meronts) had less expressed tubular appendages on the surface and exhibited closer contacts with mitochondria and intracellular filaments (Fig. 1D). In most cases though meronts could not be differentiated from the late proliferative stages (sporonts) by their morphology. We also did not detect signs of meiosis in parasite cells. At the end of the proliferation cycle, sporonts produced two sporoblasts (Fig. 1F, G and Fig. S3c, d) which matured into spores (Fig. 1H–J). All these observations are concordant with previous studies on *A. algerae* in different hosts and cell lines (Cali et al. 2004, 2005; Canning and Sinden 1973; Trammer et al. 1999). We often observed empty spores in vicinity of ovaries (Fig. S3e) suggesting autoinfection and potential transovarial transmission, in case a sporoplasm reaches an oocyte; however, we never saw infection in oocytes or nursery cells. Ultrastructural features of the spores, like size and shape, configuration of the polar disk and "vacuolated" (Canning and Sinden 1973) polaroplast, number of polar filament coils (9–11) and the structure of the spore envelope (Fig. 1H–G) proved identity of the studied microsporidia to the species initially isolated from *Anopheles stephensi* by Vavra and Undeen (1970) and described ultrastructurally by Canning and Sinden (1973). All these features were also characteristic for *A. algerae* developing in alternative hosts, human tissues, or cell lines (Cali et al. 2005).

The most fascinating and distinguishing feature of *A. algerae* development within all known host cells is formation of "vesicular-tubular structures" (Cali et al. 2004, 2005) on the surface of proliferative stages. These structures which we call here just "tubular structures" (since no vesicles are involved), were observed on the surface of nearly all stages. They often formed ridges of electron dense material on the surface of proliferative stages and sporoblasts (Fig. 2A, B). Tubules may extend into the host cytoplasm and interact with host organelles—mitochondria (Fig. 1D) and myofilaments (Fig. 2C). Eventually, the tubular structures detach from the parasite surface and fill the cytoplasm of the infected adipocyte or myocyte (Fig. 2D), often concentrating on the periphery of the latter (Fig. 2E). Another type of *A. algerae* secretion was associated with "protoplasmic extensions" (Cali et al. 1998)—elongated pseudopodia-like processors that derived from the surface of proliferative stages and sporoblasts, and were incrusted with tubular structures (Fig. 1E, F, 2F and Fig. S3d). The protoplasmic extensions were first described by Cali et al. (1998) as a characteristic feature of *Anncaliia (Brachiola) vesicularis*. They were later observed in *A. azovica* (Tokarev et al. 2018). Our current study proves that *A. algerae* stages also exhibit these processors which are, likely, common to all (or at least several) *Anncaliia* spp., but can be easily overseen during EM analysis. If so, the difference between *A. algerae* and *A. vesicularis* becomes less obvious. However, only detailed comparative molecular and morphological analysis, currently impossible, would

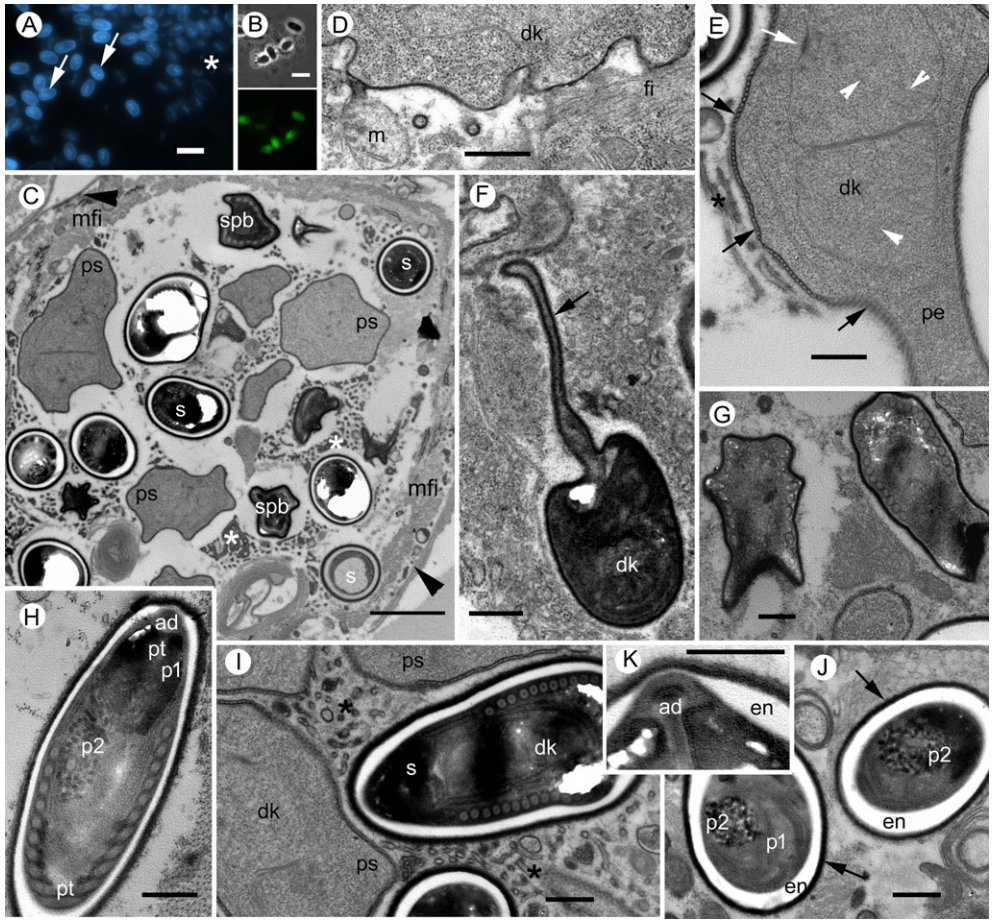

Figure 1 *Anncaliia algerae* in *Drosophila melanogaster*: light and electron microscopy. **A.** Spores stained by combination of DAPI and Calcofluor, epifluorescence: chitin components of spore walls bind Calcofluor, so the envelopes are stained in mature spores (arrows). In other stages, only diplokarya labeled with DAPI bare fluorescence (asterisks). **B.** Spores on the smear from infected transgenic fly expressing E-cadherin-GFP tag: live spores under phase contrast optics (upper image), epifluorescence in GFP channel (right image). Spores bare green fluorescence. **C.** Infected muscular fiber (i.e. a syncytium of 2 myocytes). *Anncaliia algerae* proliferative stages (PS), sporoblasts (Spb), spores (S), and tubular secretion (white asterisk) are seen in the center of the fiber. A few myofibrils (mfi) are retained at the periphery near the basal membrane (arrowhead) of the fiber. **D.** Section through an early proliferative stages with short surface tubules in adipose tissue in vicinity of ovariole**.** Tubules contact a mitochondrium (M) and intracellular fibrils (fi). **E.** A diplokaryotic proliferative stage enclosed in thickened plasmalemma ornamented with tubular structures (black arrows). Some tubules are detached from the parasite surface (asterisk). The cell forms a pseudopodia-like processor ("protoplasmic extension" (PE)). Diplokaryon (DK) is undergoing mitosis. White arrow indicates a spindle plaque, white arrowheads—mitotic microtubules. **F and G.** Sporoblasts: arrow indicates long protoplastic extension. **H.** A spore sectioned longitudinally demonstrates bipartite polaroplast with lamellar anterior (p1) and "vacuolated" posterior (p2) parts. **I.** Section through infected fiber containing a spore and proliferative stages. Sarcoplasm is filled with tubular secretion (asterisks). **J.** Transversely sectioned spores with endospore (En) and relatively thin smooth exospore free of tubular secretion (arrows). **K.** Apical end of the spore with the proximal portion of the polar filament and anchoring disc. Scale bars: A and B, 5 μm; D, 2 μm; E–J, 500 nm.

allow synonymization of these two species. In the current study, protoplasmic extensions were occasionally seen on the surface of proliferative stages and sporoblasts forming long "tails" up to 6 μm long (Fig. 1F and Fig. S3d). More often the connection of protoplasmic extensions with the mother cell was not spotted on sections (Fig. 2F and Fig. S3a, c, d). Looks like, protoplasmic extensions eventually dissociate from the mother cell and form an elaborate network inside the infected cells (Fig. 2F). The tubular structures and protoplasmic extensions ultimately fill the host sarcoplasm which gradually becomes electron lucid and void of myofilaments and other organelles. The heavily infected myofibers were composed of hypertrophied myocytes filled with numerous parasite cells, tubular structures, and the remnants of myofilaments at the periphery (Fig. 2A–F and Fig. S2c, d). Those myofibers could be easily differentiated from uninfected ones (Fig. 2G).

The ultrastructural features of host–parasite interactions observed in this study, were amazingly similar to ones

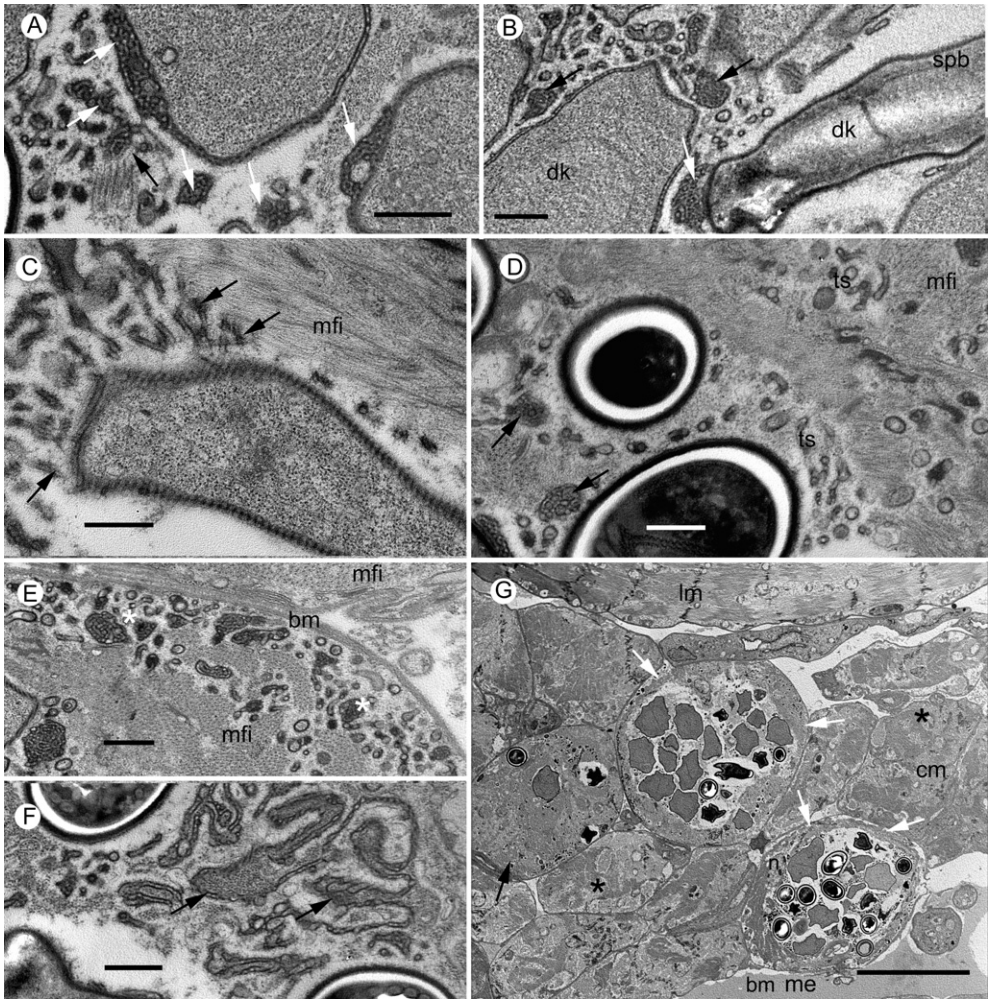

Figure 2 Ultrastructural manifestations of *Anncaliia algerae* infection in *Drosophila* visceral myocytes. **A.** Ridges of tubular structures on the surface of proliferative stages (white arrows). Black arrow points to the contact of tubules with myofibrils. **B.** Clusters of tubular structures on the sporoblast surface (a "ridge", white arrow) and in the host sarcoplasm (black arrows). **C.** A protoplasmic extension ornamented with tubular structures. Tubules extend into the host sarcoplasm and contact myofibrils (arrows). **D.** Individual tubular structures and tubular clusters (arrows) within the sarcoplasm. **E.** Tubular structures at the periphery of the infected circular fiber (asterisk). **F.** Networks formed by protoplasmic extensions (arrows) detached from their mother cells. **G.** Heavily infected myofibrils are hypertrophied and can be easily seen on sections. Black arrow points to a lightly infected fiber which is hardly distinguishable from uninfected fibers (black asterisks) at low magnification. Bars: A–F, 500 nm, G, 10 μm. bm = basal membrane; cm = circular visceral muscular fibers; dk = diplokaryon; en = endospore; lm = longitudinal visceral muscular fibers; m = mitochondrium; me = midgut epithelium; mfi = intracellular myofibrils; n = host cell nucleus; p1 = anterior part of polaroplast; p2 = posterior part of polaroplast; pe = protoplasmic extension; ps = proliferative stages; s = spores; spb = sporoblasts; ts = tubular structures.

described during human myositis caused by *A. algerae* and *A. vesicularum* (Cali et al. 1998). Such similarity suggests alike mechanisms of pathogenesis and potentially makes the *D. melanogaster—A. algerae* system a promising laboratory model for assessing effect of antimicrosporidia treatments. Interestingly, all three lineages of insect microsporidia that contain forms known to infect humans, namely *Cystosporogenes/Endoreticulatus/Vittaforma* clade, *Anncaliia/Tubulinosema* clade, and *Trachipleistophora/Vavraia* clade, include taxa of generalist pathogens as well as species tolerating high temperatures (Sokolova 2015). Such consistency may suggest specific biochemical preadaptations, genes, and regulatory factors responsible for which are yet to be identified. For example, factors analogous to LRR proteins or products of the *InterB* multigene family were hypothesized to play a role in regulating limits of host specificity within certain lineages of microsporidia (Williams et al. 2014). Given *D. melanogaster* is currently the most established genetic model, with a fully sequenced genome and easily available transgenic forms and genomic markers, a novel host–parasite system might provide new genetic tools to investigate exceptional physiological plasticity of *A.algerae* and similar generalist microsporidia that under certain conditions may infect warm-blooded animals and threaten human health.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. *Anncaliia algerae* in *Drosophila melanogaster*: light microscopy.
Figure S2. Electron microscopy: *A. algerae* in various tissues of *D. melanogaster* imagoes.
Figure S3. Electron microscopy: infected ovaries at higher magnification.
Table S1. Prevalence of *Anncaliia algerae* after experimental infection of *Drosophila melanogaster*.